

## RAPID COMMUNICATION

### Influenza A Virus Lacking the NS1 Gene Replicates in Interferon-Deficient Systems

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The NS1 protein is the only nonstructural protein encoded by influenza A virus. It has been proposed that the NS1 performs several regulatory functions during the viral replication cycle, including the regulation of synthesis, transport, splicing, and translation of mRNAs. Through the use of reverse genetics, a viable transfectant influenza A virus (delNS1) which lacks the NS1 gene has been generated. Our results indicate that the NS1 of influenza A virus is an auxiliary (virulence) factor which plays a crucial role in inhibiting interferon-mediated antiviral responses of the host. © 1998 Academic Press

Influenza A viruses are important human pathogens belonging to the group of *Orthomyxoviridae*. Their genome consists of eight different segments of negative polarity which encode 10 viral proteins. All of the proteins are structural proteins except for the NS1. The NS1 protein is an RNA-binding protein which has been implicated in a number of regulatory functions during influenza virus infection: (a) It inhibits host mRNA polyadenylation, which may contribute to the virus-induced shutoff of host protein synthesis (1). (b) It binds to the poly(A) tails of mRNAs and thus inhibits their nuclear export (2, 3). (c) The NS1 has also been shown to inhibit pre-mRNA splicing (2, 4) apparently by specifically binding U6 and U6atac snRNAs, which are key components of the spliceosome (5, 6). (d) Binding of NS1 to dsRNA prevents *in vitro* activation of the IFN-induced kinase PKR suggesting a role of NS1 in preventing IFN-mediated antiviral responses (7). (e) The NS1 protein has been implicated in regulation of viral RNA polymerase activity (8, 9). (f) It has also been shown that the NS1 is able to stimulate the translation of specific viral mRNAs (10, 11). (g) Finally, 3 host cell proteins, 2 nuclear and 1 cytoplasmic, have been reported to interact with the viral NS1 protein (1, 12, 13). Most of the proposed functions of the NS1 have been shown using *in vitro* systems or expressing the protein in mammalian cells from a plas-

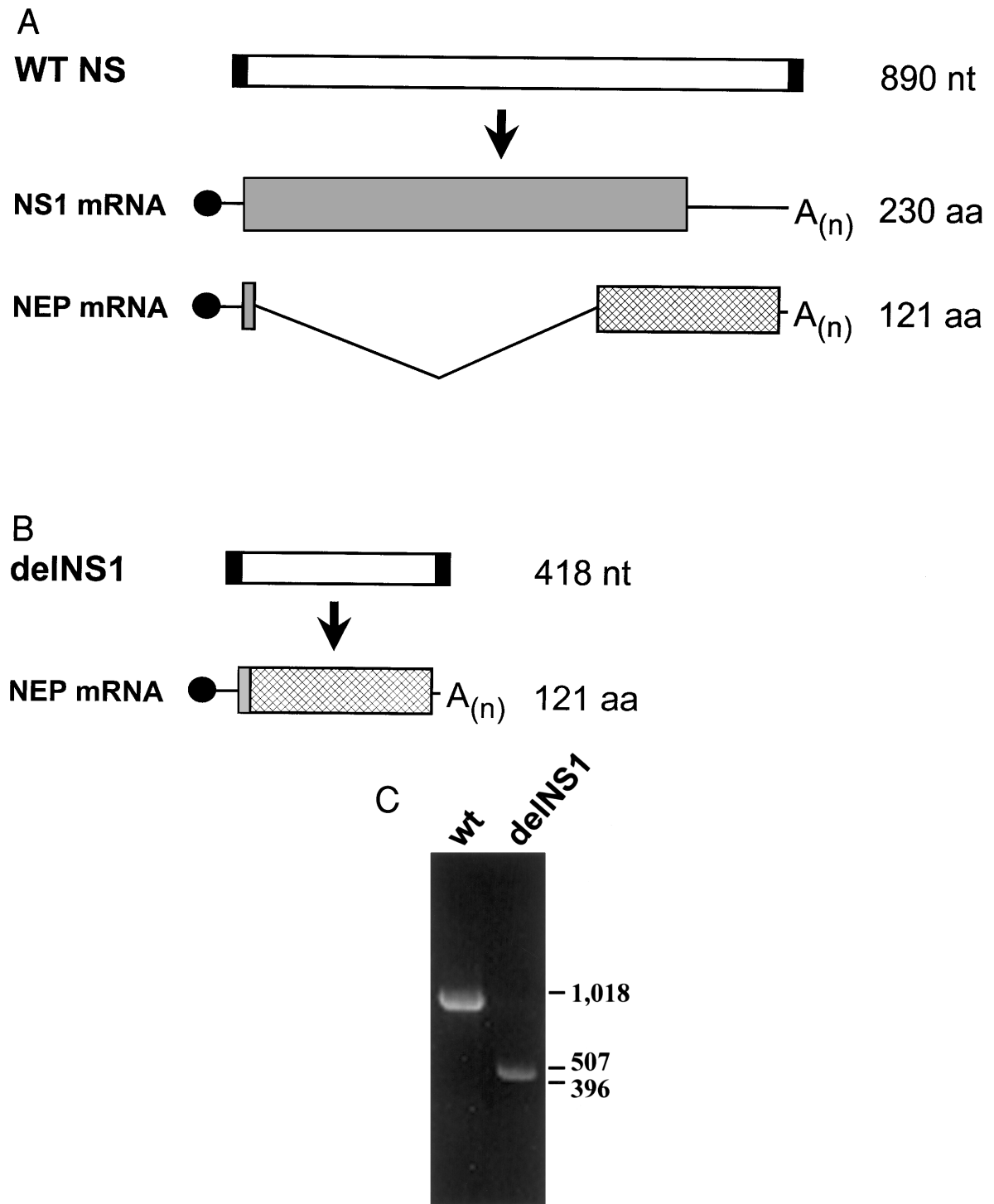
mid. In this communication, we describe the generation of a recombinant influenza A/PR/8/34 virus lacking the NS1 gene. This virus, delNS1, has a deletion in the NS gene which eliminates the NS1 open reading frame except for the first 10 amino acids which are shared with the viral NEP (NS2) protein. The characterization of the biological and molecular properties of the recombinant delNS1 virus demonstrates that the NS1 protein of influenza A virus is dispensable for viral replication in substrates deficient in the IFN pathway, suggesting that this protein is a virally encoded inhibitor of the IFN-mediated antiviral responses.

#### Generation of the transfectant influenza virus delNS1, lacking the NS1 gene

The NS-specific viral RNA segment of influenza A virus encodes both the NS1 and the NEP proteins (14). Unspliced NS-specific mRNA translates into the NS1 protein, while the spliced RNA directs the synthesis of the NEP. We have constructed plasmid pT3delNS1 which expresses a mutated NS gene from influenza PR8 virus. This mutated RNA segment contains a deletion of the NS1-specific open reading frame (nt positions 57 to 528 of the PR8 NS gene) and thus it encodes only the NEP (Fig. 1). RNP transfection of the delNS1 gene using the ts 25A-1 helper virus yielded a progeny virus which was able to grow at 40°C in Vero cells. Amplification of the NS gene of the rescued virus by RT-PCR confirmed the substitution of the NS gene of the helper virus with that derived from the transfected delNS1 gene (Fig. 1C).

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**FIG. 1.** Rescue of a transfectant influenza A virus (delNS1) lacking the NS1 gene. Schematic representations of the NS genes and NS-specific mRNAs of (A) wild-type influenza A/PR/8/34 virus (WT NS) and (B) transfectant delNS1 influenza virus are shown. Genomic RNA segments are represented as white boxes flanked by black squares. The latter represent the noncoding regions of the gene. NS-specific mRNAs are also represented. Thin lines at the ends of the mRNAs represent untranslated regions. 5' cap structures (black circles) and poly(A) tails in the mRNAs are shown. The open reading frame of the NS1 protein is represented as a gray box. The specific-NEP open reading frame is shown as a hatched box. The NEP mRNA derived from the wild-type NS gene is a spliced product of the NS1 mRNA, as indicated by the V-shaped line. (C) RT-PCR analysis of the NS RNA segment of delNS1 transfectant virus. The NS viral RNA from purified influenza A/PR/8/34 virus (wt) or from delNS1 virus (delNS1) was amplified by coupled RT-PCR using the oligonucleotide primers described under Material and Methods. The PCR products were run on a 2% agarose gel and stained with ethidium bromide. The positions of size markers are indicated on the right.

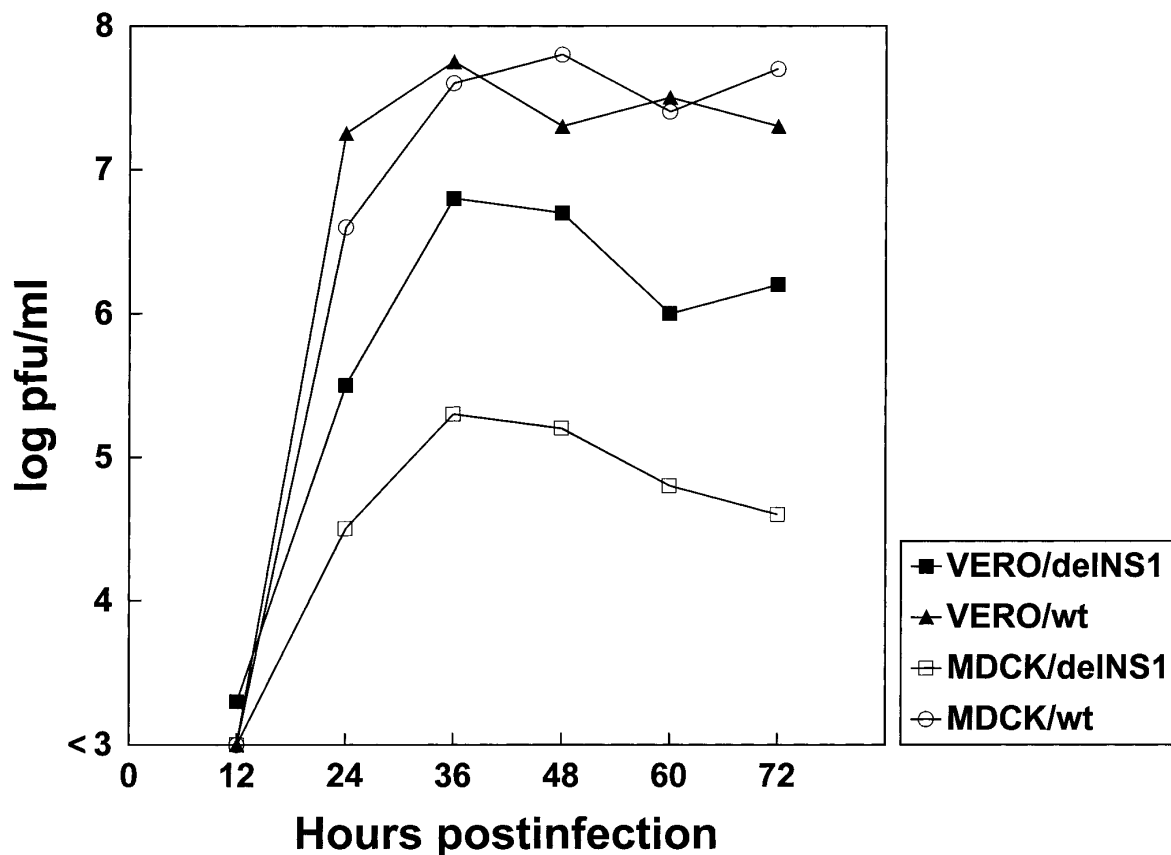


FIG. 2. Growth curves of delNS1 and PR8 viruses on Vero and MDCK cells. Confluent cell monolayers in 35-mm-diameter dishes were infected with wild-type (wt) influenza A/PR/8/34 virus and with the transfectant delNS1 virus at an m.o.i. of 0.001. At the indicated time points, infectious particles present in the media were titrated by plaque assay in Vero cells.

### Growth properties of delNS1 virus in tissue culture and eggs

We compared the growth properties of delNS1 virus and wild-type PR8 virus in Vero cells, MDCK cells, and 11-day-old embryonated chicken eggs. Cell monolayers containing  $10^6$  Vero or MDCK cells were infected with delNS1 virus or PR8 virus at multiplicity of infection (m.o.i.) of approximately 0.001. Viral titers in the supernatants were measured by plaque assay on Vero cells at 12-h intervals during 3 days. DelNS1 virus was able to grow in Vero cells to titers of  $6 \times 10^6$  PFU/ml as compared to a titer of  $6 \times 10^7$  PFU/ml for wild-type PR8 virus (Fig. 2). However, delNS1 virus replication was severely reduced in MDCK cells and in 11-day-old embryonated eggs. As shown in Fig. 2, delNS1 virus was only able to grow to titers around 3 logs lower than those of wild-type PR8 virus in MDCK cells. In addition, while PR8 virus grew to hemagglutination (HA) titers of 2048 in embryonated eggs, no HA titers were detected in eggs following inoculation of  $10^4$  PFU of delNS1 virus (3 days at  $37^\circ\text{C}$ ). When a wild-type NS gene was reintroduced by transfection in the delNS1 virus, the new "wild-type" virus was able to grow in MDCK cells and in embryonated eggs to titers similar to those of PR8 virus (data not shown). This

result demonstrates that the absence of the NS1 gene is responsible for the altered growth properties of the delNS1 virus.

### Transfectant delNS1 virus is pathogenic in STAT1 $^{-/-}$ mice

The reason for the differences between Vero cells, MDCK cells, and embryonated eggs in supporting delNS1 virus replication and viral protein expression may relate to the inability of Vero cells to synthesize IFN (15). In order to test this hypothesis, the ability of delNS1 virus to replicate and cause disease in inbred mice and in mice deficient in IFN responses was investigated. For this purpose, we used  $5 \times 10^4$  PFU of delNS1 virus to infect intranasally (i.n.) five C57BL/6 mutant mice which were homozygous for a targeted deletion of STAT1, a transactivator required for IFN signaling (16, 17). Five STAT1 $^{+/+}$  C57BL/6 and four STAT1 $^{+/+}$  BALB/c mice were also inoculated with delNS1 virus. At day 4 postinfection, two STAT1 $^{-/-}$  and two STAT1 $^{+/+}$  C57BL/6 mice were sacrificed and viral titers were measured in the lungs. STAT1 $^{-/-}$  mice had viral titers of  $2 \times 10^3$  and  $4 \times 10^3$  PFU/lung. However, no virus (less than  $10^2$  PFU/lung) was detected in the lungs of STAT1 $^{+/+}$  mice.

TABLE 1

Survival of Mice Following delNS1 Virus Infection<sup>a</sup>

Mice	Day postinfection		
	4 day	7 day	14 day
STAT1 <sup>-/-</sup> C57BL/6	5 of 5	0 of 3 <sup>b</sup>	0 of 3
STAT1 <sup>+/+</sup> C57BL/6	5 of 5	3 of 3 <sup>b</sup>	3 of 3
STAT1 <sup>+/+</sup> BALB/c	4 of 4	4 of 4	4 of 4

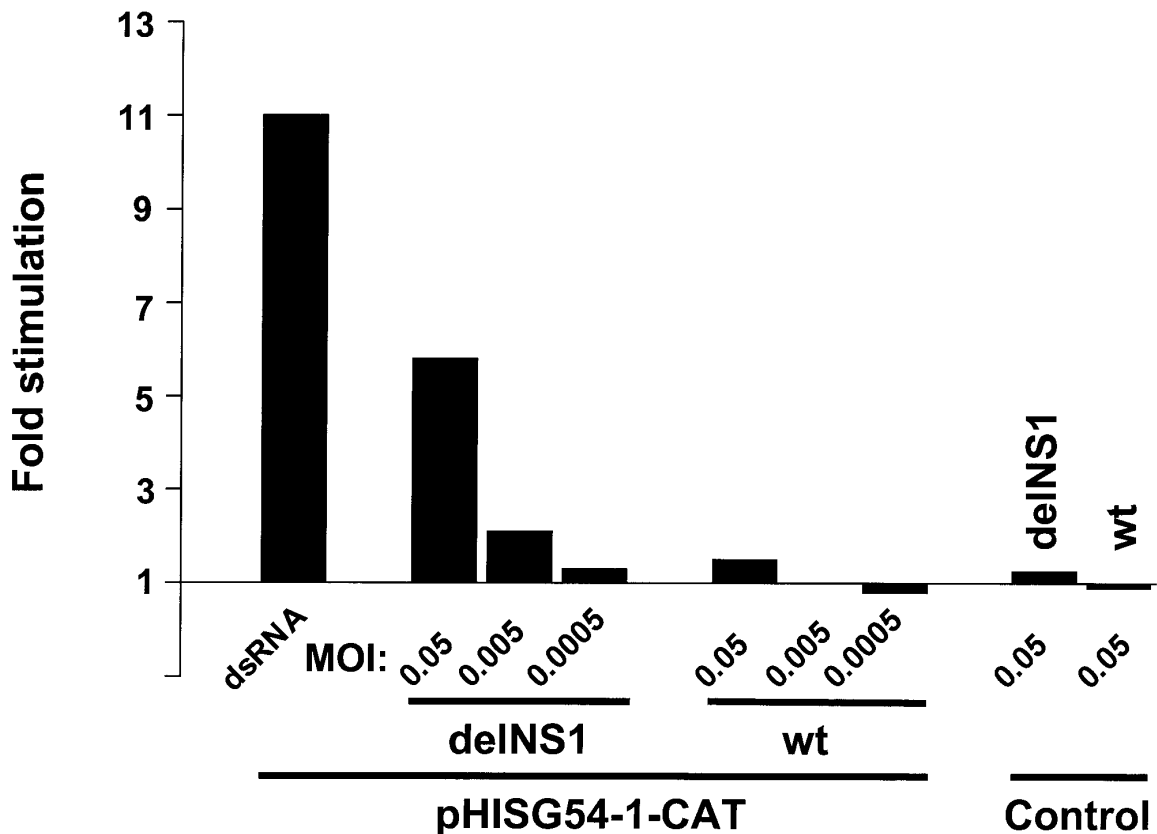
<sup>a</sup> Mice under ether anesthesia were inoculated intranasally with  $5 \times 10^4$  PFU of delNS1 virus.

<sup>b</sup> Two mice were sacrificed at day 4 to determine viral lung titers.

By day 7 postinfection, all three remaining STAT1<sup>-/-</sup> mice died (Table 1). However, all STAT1<sup>+/+</sup> mice survived infection with delNS1 virus (Table 1) without showing any symptoms of disease, as monitored by body weight loss (data not shown). It should be noted that wild-type PR8 virus is highly pathogenic for both STAT1<sup>+/+</sup> and STAT1<sup>-/-</sup> mice and replicates to titers of  $10^4$  to  $10^5$  PFU/lung in the lungs of both types of animals (18).

### Stimulation of transcription from an IFN-regulated promoter by infection with transfectant delNS1 virus

In order to investigate if the deletion of the NS1 gene in delNS1 virus results in an enhanced IFN response in infected cells we performed transfection experiments using pHISG54-1-CAT. This plasmid contains the CAT reporter gene driven by the IFN $\alpha$ -stimulated promoter of the ISG-54 K gene (19). As described under Materials and Methods, 293 cells were transfected with pHISG54-1-CAT and infected with delNS1 virus or wild-type PR8 virus at the indicated m.o.i.s. CAT activity in the infected cell extracts was compared with that in uninfected cell extracts. For positive control, transcription from the IFN-regulated promoter was stimulated using poly(I-C) as a source of dsRNA. No effect in reporter gene activity was found when cells were transfected with pCAT3-control vector containing the CAT gene under the control of an SV40 promoter and infected at an m.o.i. of 0.05 with delNS1 or wild-type virus (Fig. 3). However, infection at an m.o.i. of 0.05 with delNS1 virus, but not with wild-type virus, induced an approximately sixfold stimulation of the reporter gene under the control of the IFN-regulated



**FIG. 3.** Induction of transcription from an IFN-stimulated promoter by infection with delNS1 virus. Monolayers of 293 cells were transfected with plasmid pHISG54-1-CAT encoding the reporter gene CAT under the control of a type I IFN-stimulated promoter or with pCAT3-control vector. One-day posttransfection, cells were transfected with 50  $\mu$ g of dsRNA or infected with delNS1 virus or with wild-type influenza A/PR/8/34 virus (wt) at the indicated m.o.i.s. One-day postinfection, CAT activity was determined in cell extracts. The stimulation of CAT activity following the different treatments is indicated.

promoter (Fig. 3). The transfectant virus lacking the NS1 gene was thus impaired in its ability to inhibit the IFN response mounted by 293 cells following infection.

## Discussion

Despite the conservation of the NS1 gene among all influenza A virus isolates, and despite the presence of an NS1 homologue in influenza B viruses, and probably also in influenza C viruses, it was possible to rescue a viable NS1-minus (delNS1) virus. Although the delNS1 virus was able to grow in Vero cells, its ability to replicate was severely reduced in MDCK cells and in 11-day-old embryonated chicken eggs. In addition, levels of viral protein synthesis during one single replication cycle were lower in MDCK cells than in Vero cells infected with delNS1 virus (data not shown). Since Vero cells are deficient in IFN expression (15), we suggest that the altered tissue culture and egg growth of delNS1 virus is due to IFN-mediated effects. The following evidence supports this hypothesis. (a) Infection with delNS1 virus (m.o.i. of 0.05), but not with wild-type virus, induced transactivation of an IFN-stimulated reporter gene in 293 cells. (b) The delNS1 virus was able to replicate and to induce disease in mice that were deficient in IFN signaling, i.e., STAT1<sup>-/-</sup> animals, but the virus was nonpathogenic in control mice. These results taken together strongly suggest that one of the functions of the NS1 protein of influenza A virus is to inhibit IFN-mediated antiviral responses of the host.

A possible mechanism by which the NS1 inhibits the IFN-mediated antiviral responses is by binding (and sequestering) dsRNA (20, 21). Influenza virus-infected cells produce large amounts of negative sense RNA (vRNA) as well as positive sense RNA (cRNA and mRNA) that can form dsRNA. The presence of dsRNA has been shown to lead to IFN secretion, resulting in the activation of transcription of IFN-stimulated genes, including the genes coding for the protein kinase PKR and the enzyme 2',5'-oligoadenylate synthetase. In addition, both PKR and 2',5'-oligoadenylate synthetase are potently activated by dsRNA at a posttranslational step, resulting in inhibition of translation and in RNA degradation, respectively (22). Interestingly, it has been reported that the NS1 protein prevents the activation of PKR by dsRNA *in vitro* (7). It is also noteworthy that an influenza virus containing a truncated NS1 protein of only 80 amino acids, but not of 38 amino acids, is able to replicate in MDCK cells (23). These findings suggest that only the amino-terminal RNA-binding domain of the NS1 protein is required for the inhibition of the IFN-mediated responses.

The importance of type I IFN as a potent host response against viruses is illustrated by the fact that—as a countermeasure—many viruses express IFN antagonists. Examples include VA RNAs of adenoviruses, the Epstein-Barr virus-encoded structural small RNAs, the

K3L and E3L gene products of vaccinia virus, the NSP3 gene product of group C rotavirus, the  $\sigma 3$  protein of reovirus, the  $\gamma(1)34.5$  protein of HSV-1, and the NS5A protein of hepatitis C virus, among others. Interestingly, several of these viral products, like the NS1 protein of influenza A virus, are able to bind to dsRNA and thus prevent the activation of PKR (24, 25).

In addition to the inhibition of IFN-mediated responses, other functions have been proposed for the NS1 protein of influenza viruses. Some of these functions—such as the NS1-mediated stimulation of viral mRNA translation (10, 11)—may result from indirect effects caused by the IFN inhibition. On the other hand, the role of NS1 in inhibiting host mRNA polyadenylation, mRNA nuclear export, and mRNA splicing may be due to the direct interaction of NS1 with cellular components involved in these processes (1–3, 5, 6, 13). Although the deletion of the NS1 gene did not have dramatic effects on the replication of the virus in Vero cells, the inability of the NS1 knockout virus to perform some of the accessory functions described above might be responsible for the small (one log) difference in growth between delNS1 and PR8 virus in Vero cells. In addition, the delNS1 virus not only lacks the open reading frame for the NS1 protein but it also expresses the NEP protein from an unspliced mRNA (Fig. 1B). The fact that the delNS1 virus is viable indicates that a mechanism of regulation of NEP expression based on splicing of the NS mRNA is not required for the replication of the virus. However, at this point we have not examined the precise time course for the expression of the NEP or the M2 proteins; thus, we cannot exclude the possibility that the differential expression of viral gene products other than the NS1 (partially) contributes to the altered phenotype of the virus. Further experimentation will be required to clarify these aspects of the replication cycle of the influenza virus.

Negative-strand RNA viruses have compact genomes encoding a small number of viral proteins. Only recently has it been possible to demonstrate that some of the coding genes of this group of viruses are not essential. For example, the use of reverse genetics techniques has allowed the generation of replication-competent negative-strand RNA viruses lacking specific genes, such as the genes encoding the C proteins of measles and vesicular stomatitis viruses (26, 27), the V proteins of measles and Sendai viruses (28, 29), the SH protein of respiratory syncytial virus (30), and the NS1 protein of influenza A virus (this article). If proven to be adequately attenuated and immunogenic *in vivo*, specific “gene knockout” viruses could be used as live vaccines in the future. Also, we postulate that some (or all) of the negative-strand RNA viruses possess an auxiliary IFN antagonist.

The genetic manipulation of the NS gene of influenza A viruses may also help in generating viral vaccine vectors which express novel antigens and/or polypeptides

(37). Since the NS RNA segment is the shortest among the eight viral RNAs, it is possible that the NS RNA will tolerate longer insertions of heterologous sequences than other viral RNAs. Moreover, the NS RNA segment directs the synthesis of high levels of protein in infected cells, suggesting that it would be an ideal segment for the insertion of foreign antigens. However, further experimentation is required to determine the ability of the NS gene of influenza A viruses to support the expression of foreign proteins.

Our results suggest that the IFN-antagonist function of the NS1 protein of influenza A virus is one of the factors involved in virus pathogenicity. It is thus possible that highly pathogenic strains of influenza A virus have evolved NS1 proteins which are more efficient in preventing the IFN-mediated antiviral effects. In this respect, we have recently shown that replication of wild-type influenza A/WSN/33 virus is limited to the lungs of mice by type I IFN, and that the virus becomes pantropic in mice which are deficient in the IFN pathway (18). Thus, one could speculate that influenza viruses containing "better" NS1 proteins would be more virulent. An example of a virus with a strong IFN-antagonist might be the H1N1 virus of the 1918 influenza pandemic. The presence of a suboptimal NS1 protein in different influenza virus strains would also explain why in some instances influenza viruses appear to be good inducers of IFN (32). Moreover, influenza viruses lacking the NS1 gene, such as the delNS1 virus, may be even better IFN inducers and thus could be used therapeutically to stimulate an antiviral state against a variety of different infectious agents. Finally, the function of the NS1 protein of influenza A virus as an IFN-antagonist might be a good target for novel antiviral compounds.

## Materials and methods

**Viruses, cells, and animals.** Influenza A/PR/8/34 (PR8) virus (H1N1) was propagated in 11-day-old embryonated chicken eggs at 37°C. Influenza A virus 25A-1, a reassortant virus containing the NS segment from the cold-adapted strain A/Leningrad/134/47/57 and the remaining genes from PR8 virus (33, 34), was grown in Vero cells at 34°C. The 25A-1 virus is *ts* in mammalian cells and was used as helper virus for the rescue of the delNS1 transfectant virus. Vero cells and MDCK cells were used for influenza virus growth in the presence of 1 µg/ml of trypsin (Difco Laboratories, Detroit, MI). In plasmid transfection experiments 293 cells were used. C57BL/6 mice homozygous for a targeted deletion of STAT1 were generated as previously described (16). Specific-pathogen-free C57BL/6 and BALB/c mice were purchased from Taconic Farms.

**Animal infections.** Female mice were used for influenza virus infection at 4 to 6 weeks of age. i.n. inoculations were performed in STAT1+/+ and STAT1-/- mice

under ether anesthesia using 50 µl of MEM containing varying amounts of delNS1 virus. Animals were monitored daily and sacrificed when observed in extremis. All procedures were in accord with NIH guidelines on care and use of laboratory animals.

**Plasmids.** pT3delNS1 was made as follows. First, pUC19-T3/NS PR8, containing the complete NS gene of PR8 virus flanked by the T3 RNA polymerase promoter and a *Bpu*AI restriction site (23), was amplified by inverse PCR using primers 5'-CTGAAAGCTTGACACAGTGTG-3' and 5'-GACATACTGCTGAGGATGTC-3' (CODON Genetic Systems, Weiden, Austria). The obtained cDNA was phosphorylated, Klenow treated, self-ligated, and propagated in *Escherichia coli* strain TG1. The construct obtained after purification was named pT3delNS1 and verified by sequencing. pHISG54-1-CAT (19) and pCAT3-control vector (Promega, Madison, WI) encoded the CAT reporter gene under the transcriptional control of the IFN $\alpha$ -stimulated promoter of the ISG-54 K gene and of the SV40 promoter, respectively.

**Generation of transfectant viruses.** Generation of delNS1 virus was performed by ribonucleoprotein (RNP) transfection (35). The RNPs were formed as previously described (36). RNP complexes were transfected into Vero cells which were previously infected with 25A-1 virus. Transfected cells were incubated for 18 h at 37°C, and the supernatant was passaged twice in Vero cells at 40°C and plaque purified three times in Vero cells. The isolated delNS1 virus was analyzed by RT-PCR using two primers, one complementary to positions 1 to 21 at the 3' noncoding end of the NS gene, and the other containing the last 38 nucleotides of the 5' noncoding end of the NS gene.

**CAT transfections.** Monolayers of 293 cells in 35-mm dishes were transfected with 1 µg of pHISG54-1-CAT or 1 µg of pCAT3-control vector using DOTAP lipofection reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. One-day post-transfection, cells were infected with delNS1 virus or PR8 virus at the indicated multiplicities of infection. As controls, cells were mock-infected or transfected with 50 µg of poly(I-C) (Sigma, St. Louis, MO). After 24 h at 37°C, cell extracts were made and assayed for CAT activity, as described (35).

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